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P1 Trisaccharide (Galα1,4Galβ1,4GlcNAc) Synthesis by Enzyme Glycosylation Reactions Using Recombinant *Escherichia coli*

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The frequency of *Escherichia coli* infection has lead to concerns over pathogenic bacteria in our food supply and a demand for therapeutics. Glycolipids on gut cells serve as receptors for the Shiga-like toxin produced by *E. coli*. Oligosaccharide moiety analogues of these glycolipids can compete with receptors for the toxin, thus acting as antibacterials. An enzymatic synthesis of the P1 trisaccharide (Galα1,4Galβ1,4GlcNAc), one of the oligosaccharide analogues, was assessed in this study. In the proposed synthetic pathway, UDP-glucose was generated from sucrose with an *Anabaena* sp. sucrose synthase and then converted with an *E. coli* UDP-glucose 4-epimerase to UDP-galactose. Two molecules of galactose were linked to *N*-acetylglucosamine subsequently with a *Helicobacter pylori* β-1,4-galactosyltransferase and a *Neisseria meningitidis* α-1,4-galactosyltransferase to produce one molecule of P1 trisaccharide. The four enzymes were coexpressed in a single genetically engineered *E. coli* strain that was then permeabilized and used to catalyze the enzymatic reaction. P1 trisaccharide was accumulated up to 50 mM (5.4 g in a 200-ml reaction volume), with a 67% yield based on the consumption of *N*-acetylglucosamine. This study provides an efficient approach for the preparative-scale synthesis of P1 trisaccharide with recombinant bacteria.

Recently, vaccines have been introduced to prevent illness from pathogenic *Escherichia coli*, yet there remains a need for therapeutics to treat acute infection (27). Unlike endogenous *E. coli* from our intestine, the pathogenic strain (O157:H7) has acquired a gene from *Shigella* which produces a toxic protein. This Shiga-like toxin binds to glycolipid receptors on cells of the gut wall and can ultimately lead to dysentery, hemorrhagic colitis, and sometimes life-threatening complications (32). The attachment of the microbial protein onto mammalian cells through surface carbohydrates initiates a successful infection (18). The Shiga-like toxin will also bind other carbohydrate derivatives, including globotriosylceramide (Galα1,4Galβ1,4Glc-ceramide) found on the cell surface (4, 24–26, 44) and trisaccharide analogues, such as globotriose (Galα1,4Galβ1,4Glc) and the trisaccharide moiety of P1 antigen (Galα1,4Galβ1,4GlcNAc) (1). The mechanism of action for the proposed therapy involves preventing the toxin from binding to cell receptors by introducing these molecules to the site of infection. Instead of binding to cell receptors, the toxic peptides bind to the carbohydrate analogues and are removed from the body without causing harm. Of the three carbohydrate moieties mentioned above, P1 trisaccharide is the most effective therapeutic. Although synthesis remains a limiting factor, if available in large quantities these trisaccharides have considerable potential as antiadhesive agents in the treatment of pathogenic *E. coli* infections (17, 36, 37).

Chemical syntheses of oligosaccharides have always been complicated by their structural complexity and have traditionally involved laborious protection protocols. Having been developed significantly in recent years, biocatalytic approaches to oligosaccharide synthesis offer many advantages over traditional methods (15, 34). Glycosyltransferases, as well as glycosidases (29, 42), or whole cells of metabolically engineered bacteria have been applied in the synthesis of oligosaccharides and analogues (5, 8, 35). To date, catalysis with Leloir's glycosyltransferases (19, 47) as well innovations in the regeneration of glycosylation donors have greatly advanced the field (3, 16, 46, 48, 49). Biocatalytic synthesis, however, still requires the tedious purification of the individual enzymes involved in catalysis and careful stoichiometric balancing of high-energy phosphates. Kyowa Hakko, Inc., in Japan, is one of several companies that have accomplished large-scale syntheses of sugar nucleotides and oligosaccharides (21, 22, 33, 50). In such experiments, several recombinant bacterial strains expressing enzymes involved in the catalytic pathway of particular carbohydrates were coupled. The development of a metabolically engineered strain of *Corynebacterium ammoniagenes* with high-level UTP production and UDP-galactose (UDP-Gal) regeneration was a key contribution to the present work (11–13, 20). Subsequently, an alternate pathway for UDP-Gal formation from sucrose and UDP has been developed. With a catalytic amount of UDP, sucrose synthase (SusA) catalyzes the cleavage of sucrose to release UDP-glucose (UDP-Glc) and fructose. UDP-Glc-4-epimerase (GalE) catalyzes the conversion from UDP-Glc to UDP-Gal. Both are reversible reactions driven forward by constant consumption of UDP-Gal in glycosylation (51). We have previously reported work involving the synthesis of trisaccharides using a single recombinant *E. coli* strain harboring three enzymes: SusA, GalE, and bovine α-1,3-galactosyltransferase (α1,3GalT). Using lactose as the acceptor in this system, the α-Gal epitope (Galα1,3Galβ1,4Glc) was synthesized. When α1,3GalT was replaced with the *Neisseria meningitidis* α1,4GalT (LgtC), the resulting oligosaccharide was modified to the globotriose (Galα1,4Galβ1,4Glc) (7).

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In this paper, a fourth enzyme was added to the previous three (7), β1,4GalT from *Helicobacter pylori*. The corresponding gene possesses a poor homology with *lgtB*, another β1,4GalT gene found in *N. meningitidis* (45). By transferring a galactosyl to *N*-acetylglucosamine (GlcNAc), β1,4GalT catalyzes the synthesis of *N*-acetyllactosamine (LacNAc;Galβ1,4GlcNAc) (10). LacNAc is then combined with an additional galactosyl by α1,4GalT, resulting in the synthesis of the P1 trisaccharide (Galα1,4Galβ1,4GlcNAc). The focus of our study is the development of large-scale production of this P1 trisaccharide with the inclusion of UDP-Gal regeneration in a single genetically engineered bacterium.

MATERIALS AND METHODS

Plasmids and bacterial strains. The plasmid vector pLDR20 (ATCC 87205), *E. coli* K-12 substrain MG1655 (ATCC 47076), *Anabaena* sp. strain PCC 7119 (ATCC 29151), *H. pylori* (ATCC 43504; NCTC 11637), and *H. pylori* 26695 (ATCC 700392) were purchased from the American Type Culture Collection, Manassas, Va. The plasmid vector pET15b and *E. coli* BL21(DE3) cells [F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (*DE3*)] were from Novagen, Madison, Wis. *E. coli* DH5α cells (*lacZΔM15 hsdR recA*) were from Gibco-BRL Life Technology, Rockville, Md. *E. coli* NM522 cells {*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 (r_K^- m_K^+) [*F'proAB lacI*^{q}ZΔ*M15*]} were from Stratagene, La Jolla, Calif. The chromosomal DNA of *N. meningitidis* MC58(L3) was a kind gift from Michel Gilbert (Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada).

Cloning, expression, and purification of individual enzymes. Molecular cloning was performed as described by Sambrook et al. (40). Restriction enzymes and T4 DNA ligase were obtained from Promega, Madison, Wis. As described in a previous study by Chen et al. (7), the *galE* gene from *E. coli* K-12, *susA* gene from *Anabaena* sp., and truncated *lgtC* gene (45 bp deleted from the 3′ end) from *N. meningitidis* were cloned onto pET15b. Individual enzymes were expressed as hexahistidine-tagged fusion proteins in *E. coli* BL21(DE3) cells and purified with nickel nitrilotriacetic acid agarose (Qiagen, Santa Clarita, Calif.) affinity chromatography. The gene encoding β1,4GalT was PCR amplified with *Taq* DNA polymerase (Fisher Scientific, Chicago, Ill.) from *H. pylori* (ATCC 43504; NCTC 11637) (forward primer, 5′-GAGTCTTCATGATTCGTGTTTTTATCATTTCTTTAAATC-3′; reverse primer, 5′-CGCGTCGACCTCGAGCTATACAAACTGCCAATATTTC-3′). At both ends of the PCR product, restriction sites (*Bsp*HI at the 5′ end and *Xho*I at the 3′ end) were introduced for insertion of the gene into pET15b at *Nco*I and *Xho*I sites (underlined) directly upstream of the T7 promoter. The recombinant β1,4GalT without a histidine tag was expressed in BL21(DE3) cells under the induction of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (Fisher). Bacterial cells were pelleted, resuspended, and lysed by the addition of lysozyme and sonication. Cellular debris was spun down, and β1,4GalT was purified from the supernatant by using $(NH_4)_2SO_4$ precipitation and gel filtration with a HiLoad 16/60 Superdex 200 column on a ÄKTA fast-performance liquid chromatography system (Amersham Pharmacia Biotech, Piscataway, N.J.). The expression of proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23), and their concentrations were measured using the Lowry method (28).

Enzymatic activity assays. Enzyme activity, as applied in this paper, is defined as the amount of an enzyme required to produce 1 micromole of the product per minute at 25°C. As well, protocols used to assay the activities of α1,4GalT (7) and GalE (6) were performed as published elsewhere; however, reactions were carried out at 25°C.

SusA activity during the generation of UDP-Glc from sucrose was measured indirectly by following the increase at A_{340} as NAD was reduced to NADH by UDP-Glc dehydrogenase (UDPG-DH; Sigma, St. Louis, Mo.). The reaction was performed in 800 μl of buffer (50 mM morpholineethanesulfonic acid [MES] [pH 6.0], 10 mM KCl, 10 mM $MgCl_2$, 100 mM sucrose, 5 mM UDP, 2 mM NAD, 0.05 U of UDPG-DH) and 200 μl of cell lysate or purified SusA.

β1,4GalT was assayed following a protocol similar to that for α1,4GalT (7). The reaction was performed in 80 μl of buffer (50 mM HEPES [pH 7.2], 20 mM KCl, 10 $MgCl_2$, 20 mM GlcNAc) with 0.3 mM UDP-[6-^{3}H]Gal (final specific activity, 1,000 cpm nmol^{-1}; Amersham Pharmacia) and 20 μl of cell lysate or purified enzyme. GlcNAc was omitted in the blank control. Allowed to run for 15 min at 25°C, the enzymatic reaction was terminated by adding 100 μl of ice-cold 100 mM EDTA and 800 μl of a DOWEX 1X8-200 chloride anion exchange resin (Sigma) slurry (prepared in distilled water, 1:1 [vol/vol]). After centrifugation, 0.5 ml of the supernatant was mixed with 10 ml of ScintiVerse BD (Fisher) and counted in an LS-3801 liquid scintillation counter (Beckman Coulter, Inc., Ontario, Canada).

Construction of recombinant bacteria. Using constructed plasmids (pET15b-*susA*, pET15b-*galE*, pET15b-*lgtC*, and pET15b-β*1,4GalT*), an artificial gene cluster, with ribosomal binding site sequences preceding each gene, was created by subcloning genes into pLDR20. The plasmid pLDR20 contains the PR promoter and cI857 repressor expression cassette from phage λ, providing a temperature control to a gene(s) cloned under PR. Using pLDR20-CES containing *susA*, *galE*, and *lgtC* (7), β*1,4GalT* was added to create a four-gene recombinant plasmid, pLDR20-CβES. The β*1,4GalT* gene was PCR amplified from pET15b-β*1,4GalT* with *Sal*I and *Sac*II sites at either end (forward primer, 5′-TCCCCGCGGAATAATTTTGTTTAACTTTAAG-3′; reverse primer, 5′-CGCGTCGACCTCGAGCTATACAAACTGCCAATATTTC-3′). *Sal*I and *Sac*II restriction sites were placed between *lgtC* and *galE* on pLDR20-CES for β*1,4GalT* insertion. A sequence of the ribosomal binding site, without a histidine tag, was added immediately upstream of the recloned β*1,4GalT* gene. The completed recombinant plasmid pLDR20-CβES (Fig. 1b) was used for the transformation of *E. coli* NM522 competent cells.

Synthesis of oligosaccharides. Once transformed, NM522(pLDR20-CβES) cells were incubated on fresh ampicillin (150 μg ml^{-1}) Luria-Bertani (LB) agar plates overnight at 30°C. Starter cultures (50 ml) were grown using LB medium with ampicillin (150 μg ml^{-1}) for 8 h at 30°C in an incubator-shaker (New Brunswick Scientific, Edison, N.J.). The 50-ml saturated culture was transferred into a 4-liter flask containing 1 liter of fresh LB (ampicillin concentration, 150 μg ml^{-1}). When the culture reached a suitable density (A_{600} = 1.0) after 3 to 4 h at 30°C, the expression of recombinant enzymes was activated by shifting the temperature from 30 to 40°C. After shaking at 40°C for another 3 to 4 h, bacteria were harvested by centrifugation (4,000 × *g*; 20 min). The cell paste (wet weight about 5 g per liter) was then resuspended at 0.5 g ml^{-1} in buffer (20 mM Tris-HCl [pH 7.0], 1% Triton X-100, 5 mM EDTA, and 10 mM 2-mercaptoethanol). The suspension was freeze-thawed twice to permeabilize the cells.

Oligosaccharide synthesis was performed in a reaction buffer (50 mM MES [pH 6.0], 10 mM $MgCl_2$, 4 mM dithiothreitol, 200 mM sucrose, 5 mM UDP, 10 to 200 mM GlcNAc) with a suitable amount of permeabilized cells. For the optimization of reaction conditions, multiple small-scale 1-ml reaction mixtures were set up using different substrates and 0.1 g (200 μl) of cells. Synthesis was carried out at 25°C and monitored by silica thin-layer chromatography (TLC; *i*-PrOH-NH_4OH-H_2O = 7:3:2) and MICROSORB-100 Å amino high-performance liquid chromatography (HPLC; isocratic CH_3CN-H_2O = 65:35) coupled with a UV detector (for UDP-Glc) and refractive index detector (for sucrose, GlcNAc, and P1 trisaccharide). The supernatant of the reaction solution was directly loaded on TLC. To prepare HPLC samples, 150 μl of the reaction solution was taken, mixed with 150 μl of acetonitrile, and then centrifuged to remove any precipitate before injection into the HPLC apparatus.

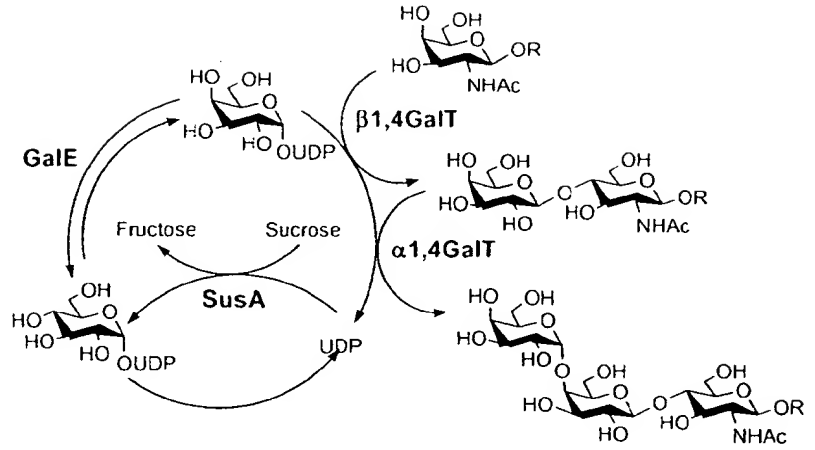
Once optimized, oligosaccharide synthesis was scaled up to a 200-ml volume (200 mM [13.7 g] sucrose, 5 mM [0.4 g] UDP-Glc, 75 mM [3.3 g] GlcNAc, and 0.1 g of permeabilized cells ml^{-1} [wet weight, 20 g]), and the reaction mixture was stirred at 25°C for 72 h (or 96 h for the time course analysis).

Purification of oligosaccharides. Enzymatic synthesis was terminated by placing the reaction mixture in a hot water bath (100°C) for 10 min. The supernatant of the reaction mixture was collected by centrifugation (10,000 × *g*; 30 min) and adjusted to pH 5.0 to 6.0. Unincorporated sucrose was thoroughly degraded by treating the supernatant with 10 mg of invertase (5,000 U; Sigma) for 24 h at 25°C. Oligosaccharides generated during synthesis were removed from solution by absorption to 70 g of activated charcoal (Sigma). Charcoal was rinsed to remove remaining monosaccharides with 2 liters of H_2O. Disaccharides and completed trisaccharides were eluted with 10% ethanol and 15% ethanol, respectively. If required, the disaccharide was enzymatically removed from the trisaccharide fraction with β-galactosidase. Using TLC to monitor fractionation, the trisaccharide was purified using Bio-Gel P-2 size-exclusion chromatography (1.5 by 120 cm; Bio-Rad, Richmond, Calif.) and lyophilized (Virtis, Gardiner, N.Y.).

Galβ1,4GlcNAc. ESI-MS: 405.98 (M + Na^+), 428.00 (M + 2 Na^+ − H^+), 788.88 (2 M + Na^+) 810.84 (2 M + 2 Na^+ − H^+). ^{1}H-NMR (D_2O, 500 MHz) δ 5.07 (*d*, 0.6H, H-1α, *J* = 2.5 Hz), 4.58 (*d*, 0.4H, H-1β, *J* = 7.6 Hz), 4.338, 4.334 (*d*, 1H, H-1′, *J* = 8.1 Hz, 7.6 Hz), 3.38-3.85 (*m*, 13H), 1.91 (*s*, 3H, NAc); ^{13}C-NMR (D_2O, 125 MHz) δ 174.89, 174.62, 103.10, 103.04, 95.04, 90.70, 78.97, 78.54, 75.52, 75.01, 72.68, 71.14, 70.43, 69.44, 68.72, 61.18, 60.25, 60.12, 56.38, 53.88, 22.34, 22.04.

Galα1,4Galβ1,4GlcNAc. ESI-MS: 546.02 (M + H^+), 563.03 (M + NH_4^+), 567.98 (M + Na^+), 583.93 (M + K^+), 1090.90 (2 M + H^+), 1112.89 (2 M +

a.



2 Sucrose + GlcNAc $\xrightarrow{UDP}$ Galα1,4Galβ1,4GlcNAc + 2 Fructose (1)

b.

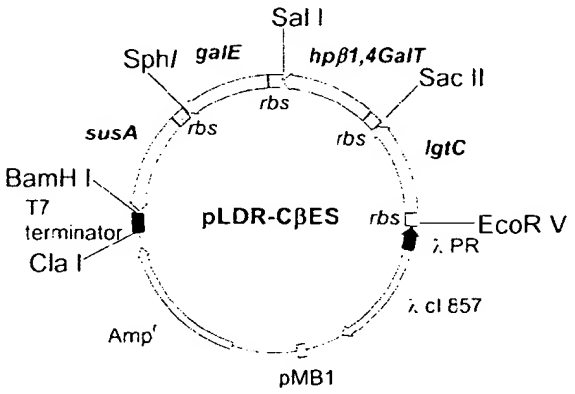


FIG. 1. (a) The biosynthetic pathway of P1 trisaccharide with the regeneration of UDP-Gal. (b) Recombinant plasmid harboring four of the involved genes.

Na^+). 1128.83 (2 M + K^+). ^{1}H-NMR (D_2O, 400 MHz) δ 5.15 (*d*, 0.6H, H-1α, *J* = 1.6 Hz), 4.89 (*d*, 1H, H-1'', *J* = 3.2 Hz), 4.67 (*d*, 0.4H, H-1β, *J* = 7.3 Hz), 4.48 (*d*, 1H, H-1', *J* = 8.1 Hz), 4.31 (*t*, 1H, H-4', *J* = 6.5 Hz), 3.50 to 4.00 (*m*, 18H), 1.99 (*s*, 3H, NAc); ^{13}C-NMR (D_2O, 100 MHz) δ 174.96, 174.70, 103.50, 100.53, 95.07, 90.76, 79.28, 78.87, 77.55, 75.66, 75.14, 72.75, 72.37, 71.15, 70.55, 69.55, 69.38, 69.16, 68.80, 60.73, 60.60, 60.27, 60.15, 56.60, 54.05, 22.44, 22.14.

RESULTS

Biosynthetic pathway. Synthesis of P1 trisaccharide, Galα1,4Galβ1,4GlcNAc, was achieved by the recombination of four enzymes. Two galactosyltransferases, *H. pylori* β1,4GalT and *N. meningitidis* α1,4GalT, as well as SusA and GalE that were used for the generation UDP-Glc from sucrose and UDP-Glc from UDP-Gal, respectively, were coupled together (Fig. 1b). One equivalent of P1 trisaccharide was produced from two equivalents of sucrose, one equivalent of GlcNAc, and a catalytic amount of UDP (Fig. 1a).

Stepwise confirmation of the proposed biosynthetic pathway was done using five reactions with different combinations of purified enzymes and starting materials (Table 1). Each catalytic step was investigated by introducing one or more enzymes to various precursors. Addition of β1,4GalT and α1,4GalT individually (Table 1, combinations 1 and 2) with UDP-Gal as a donor and GlcNAc or lactose as an acceptor yielded 90 and 95% efficiency in the production of LacNAc and Galα1,4Lac, respectively. Coupling α1,4GalT and β1,4GalT (Table 1, combination 3) with two equivalents of UDP-Gal and GlcNAc resulted in 73% efficiency in the production of P1 trisaccharide. Reduction in the yield of P1 trisaccharide was likely the result of competition between the two galactosyltransferases for UDP-Gal. With the addition of GalE (Table 1, combination 4) to the above galactosyltransferases, the generation of P1 trisaccharide remained equally efficient despite the change in substrate to UDP-Glc. The addition of SusA to the reaction allowed for the production of P1 trisaccharide from sucrose and GlcNAc precursors at an efficiency of 60%, confirming the proposed biosynthetic pathway (Table 1, combination 5).

TABLE 1. Stepwise production of the P1 trisaccharide Galα1,4Galβ1,4GlcNAc

Combination	Enzyme	Starting material	Product	Yield[a] (%)
1	β1,4GalT	UDP-Gal + GlcNAc	Galβ1,4GlcNAc[b]	90
2	α1,4GalT	UDP-Gal + lactose	Galα1,4-lactose[b]	95
3	α1,4GalT+β1,4GalT	UDP-Gal + GlcNAc	Galα1,4Galβ1,4GlcNAc	73
4	GalE+α1,4GalT+β1,4GalT	UDP-Glc + GlcNAc	Galα1,4Galβ1,4GlcNAc	75
5	SusA+GalE+α1,4GalT+β1,4GalT	Sucrose + GlcNAc	Galα1,4Galβ1,4GlcNAc	60

[a] The yield was calculated from HPLC results and the amount of lactose (Combination 2) or GlcNAc (1, 3–5).
[b] The structure was not confirmed.

Recombinant expression of enzymes. Expression of *susA*, *galE*, *lgtC*, and *β1,4GalT* from the constructed expression vector pLDR20-CβES was confirmed by SDS-PAGE (96.6 kDa for SusA, 39.8 kDa for GalE, 38.2 kDa for α1,4GalT, and 32.0 kDa for β1,4GalT [Fig. 2]). Judging from the comparable cell densities between populations of *E. coli* BL21(DE3) (pET15b-*susA*, pET15b-*galE*, pET15b-*lgtC* or pET15b-*β1,4GalT*), enzymes did not exert a toxic effect on their hosts. The expression level of each protein was also comparable (Table 2). However, solubility differed as follows: GalE was approximately 100% soluble, and α1,4GalT, β1,4GalT, and SusA were 80, 50, and 20% soluble. The insoluble portions of proteins occurred within inclusion bodies. Compared to individual expression in BL21(DE3) cells reported above, the abundance or activity of coexpressed enzymes in NM522(pLDR20-CβES) cells was reduced. Indirect evidence suggests that this observed decrease was the result of nutrient limitations rather than an intermolecular effect between recombinant enzymes.

Production of P1 trisaccharide. The molarity of GlcNAc in reaction buffer was found to influence the ratio and abundance of P1 trisaccharide and associated di- and tetrasaccharides (Fig. 3). P1 trisaccharide production was optimal at 75 mM GlcNAc. Above this concentration, synthesis began to favor the production of the disaccharide LacNAc due to the consumption of UDP-Gal by the primary galactosylation of GlcNAc. Unexpectedly, conditions of low GlcNAc lead to the formation of a tetrasaccharide (putative structure, Galα1,4Galα1,4Galβ1,4GlcNAc). Theoretically, if UDP-Gal were continuously supplied, α1,4GalT would catalyze a fourth galactosylation of the P1 trisaccharide, generating the tetrasaccharide.

When scaled up to 200 ml over a 96-h time course, the biosynthesis of P1 trisaccharide reached a maximal yield of 50 mM at 72 h (Fig. 4). Closer analysis revealed complete consumption of GlcNAc, with 67% being converted to P1 trisaccharide and the remainder going to production of di- and tetrasaccharides. Sucrose (120 mM) remained relatively abundant, while UDP-Glc was exhausted by 72 h. The depletion of UDP-Glc exceeded theoretical consumption, suggesting chemical degradation during the reaction. Purification of P1 trisaccharide from reactions yielded on average 80% recovery. As described in Materials and Methods, mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses confirmed the structural identity of LacNAc and P1 trisaccharide.

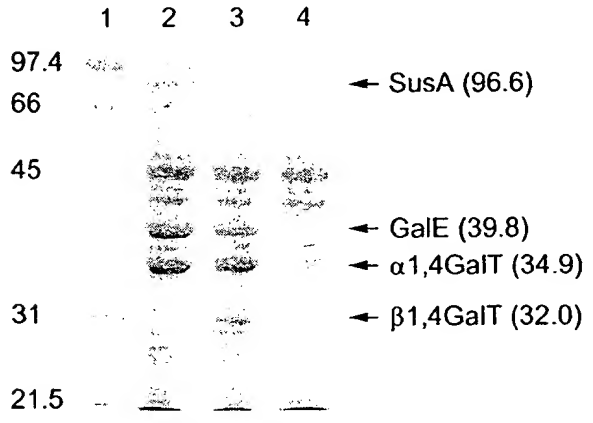


FIG. 2. SDS-PAGE (12.5%) indicating the coexpression of enzymes in recombinant bacteria. Lane 1, low-range standard proteins; lane 2, α1,4GalT, GalE, and SusA in NM522(pLDR-CES); lane 3, α1,4GalT, β1,4GalT, GalE, and SusA in NM522(pLDR-CβES); lane 4, no specific band was shown in NM522(pLDR20).

DISCUSSION

In selecting the source of SusA, several analogues from higher plants with a low K_m for sucrose and the capability of UDP-Glc generation were considered (30). However, SusA from prokaryotic *Anabaena* sp., a filamentous heterocystous cyanobacterium, was chosen because of compatibility considerations related to expression in recombinant *E. coli* (9, 38, 39). The design of this recombinant biosynthetic pathway for in vivo systems had an unforeseen advantage over in vitro systems. Fructose, which is a byproduct of the sucrose cleavage, accumulates in vitro, causing feedback inhibition (Fig. 1a). However, in vivo permeabilized bacterial cells keep fructose levels in check by consuming the fructose as a carbon source (7).

Recently, trisaccharide synthesis involving SusA (referred to as SuSy) from potato, GalE from *Streptococcus thermophilus*, human β1,4GalT, and murine α1,3GalT was reported (2). Starting from sucrose and GlcNAc, an α-Gal epitope (Galα1,3Galβ1,4GlcNAc) was synthesized in an in vitro enzymatic

TABLE 2. Activity comparison of enzymes from individual expression and coexpression

Enzyme	Solubility (%)	Individual expression (U liter^{-1})	Coexpression (U liter^{-1})
GalE	100	200	100
SusA	20	10	5
α1,4GalT	80	30	18
β1,4GalT	50	40	20

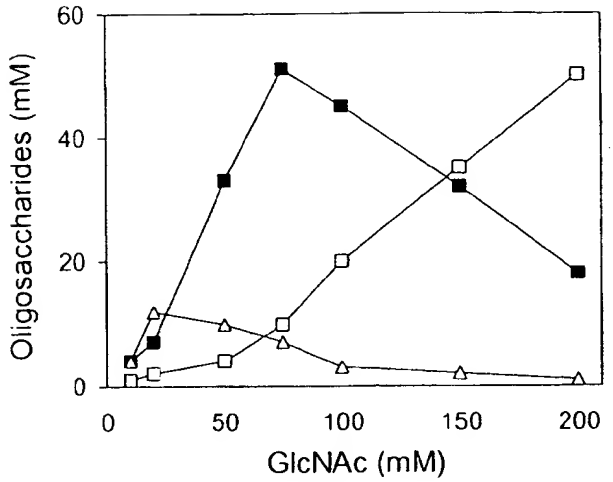


FIG. 3. Influence of GlcNAc concentration on the production of P1 trisaccharide, LacNAc, and tetrasaccharide. P1 trisaccharide is shown as closed squares, LacNAc is shown as open squares, and tetrasaccharide is shown as open triangles. The results are the mean of three experiments using 1-ml-scale reaction volumes.

reaction using individually purified enzymes. Because of interference in enzyme function, a histidine tag cannot be fused to either the N or C terminus of β1,4GalT (data not shown) and therefore must be purified without the use of affinity chromatography. Consequently, the application of recombinant bacteria to the in vivo generation of the trisaccharide represents an efficient and simpler method of synthesis.

The K_m and K_i values of human β1,4GalT for GlcNAc are 34.6 and 14.3 mM, respectively (2). Interestingly, our study found *H. pylori* β1,4GalT not to be inhibited until a much higher concentration, with activity being maintained up to 200 mM GlcNAc (Fig. 3). Also of note is the ability of GlcNAc to inhibit α1,4GalT from *N. meningitidis* at high concentrations.

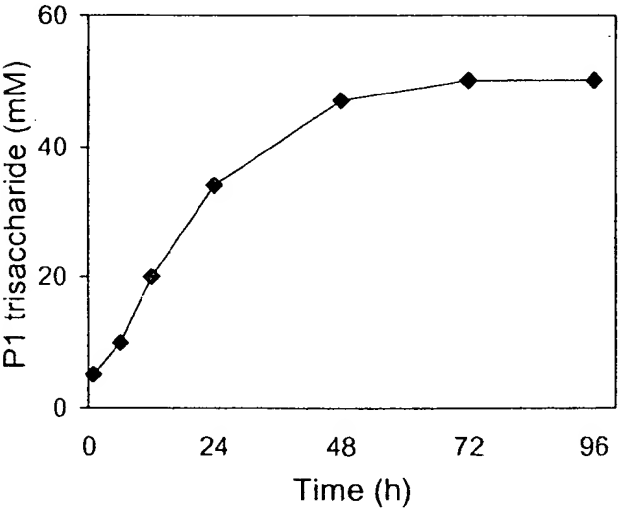


FIG. 4. Time course of P1 trisaccharide synthesis. The results are the mean of three experiments using large-scale reaction volumes (once at 200 ml, twice at 100 ml). Standard errors of the means (not shown) amounted to less than 5% of the corresponding means.

Despite GlcNAc not being the substrate of α1,4GalT, when the concentration exceeded 75 mM enzyme activity was inhibited, resulting in the accumulation of the disaccharide LacNAc (Fig. 3).

During galactosylation, the UDP portion of UDP-Gal was regenerated in situ and reused after the delivery of galactose to carry additional glucose released from sucrose cleavage (Fig. 1a). As a result of working in vivo, reactions had to be slightly modified to maintain yield. The gradual hydrolysis of UDP-Glc by cellular enzymes required the addition of slightly more than catalytic amounts of UDP-Glc for maximized P1 trisaccharide yield. Likewise, even though β-galactosidase-deficient NM522 cells were used, difficulties with substrate and enzyme degradation by cellular phosphatases and proteases required that cells be only moderately permeabilized. A protease-deficient *E. coli* strain, AD202, may provide a solution in future work; evidence suggests such strains have improved stability of recombinant enzymes (31).

In our experience, the addition of further genes to pLDR20-CβES may be limited because of spatial considerations on the plasmid. However, further development of this gene cluster for larger multiple-enzyme reactions may be possible with the use of gene fusion (e.g., *galE* and a galactosyltransferase gene). This has been attempted in other systems with success and in fact has been related to improved enzyme kinetics (6, 14).

Substrate specificity studies on different glycosyltransferases including α1,3GalT and β1,4GalT have demonstrated that a series of deoxygenated UDP-Gal derivative donors and modified acceptors can be used as substrates (15, 34, 43). Such previous literature suggested that chemical-enzymatic approaches, such as the one developed here, could act as an alternative for the rapid production of chemically modified oligosaccharide analogues. As we have demonstrated, this approach not only avoids the laborious tasks of total chemical synthesis or enzyme purification but also allows the synthesis of multiple oligosaccharides in a single step. Furthermore, the use of multiple glycosyltransferases as applied in combinatorial chemistry creates additional chemical diversity from unprotected and chemically modified substrates (41).

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